Clinical and Experimental Rheumatology 1999; 17: 343-346.

Nitrite production in mouse 3T3 fibroblasts by bleomycin-stimulated peripheral blood mononuclear cell factors

T. Yamamoto[1], I. Katayama[2], K. Nishioka[1]

[1]Department of Dermatology, Tokyo Medical and Dental University, School of Medicine, Tokyo; [2]Department of Dermatology, Nagasaki University, School of Medicine, Nagasaki, Japan

The study was supported in part by grant of Scleroderma from Japanese Ministry of Public Welfare.

Please address correspondence and reprint requests to: Toshiyuki Yamamoto, MD, Department of Dermatology, Tokyo Medical and Dental University, School of Medicine, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, Japan.

Received on September 14, 1998; accepted in revised form on December 2, 1998.

Key words:
Nitric oxide, systemic sclerosis, bleomycin, fibroblasts, peripheral blood mononuclear cells.

ABSTRACT

Objective

It is well known that bleomycin induces tissue fibrosis, including pulmonary fibrosis or scleroderma-like conditions. However, the pathogenesis has not been completely elucidated. We recently observed that peripheral blood mononuclear cells (PBMCs) stimulated by bleomycin show growth stimulatory activity for fibroblasts. Nitric oxide (NO) is an important mediator of immune and inflammatory responses, and has recently been suggested to play a role in the pathogenesis of autoimmune disorders. In this study, we have examined whether bleomycin-stimulated PBMCs induce nitrite production in mouse 3T3 fibroblasts in vitro.

Methods.

PBMCs were obtained from 6 patients with systemic sclerosis (SSc) and 6 normal volunteers, and stimulated by bleomycin; the culture supernatants were collected as conditioned medium (CM). The release of nitrite from 3T3 fibroblasts after incubation with CM was determined by Griess reagents. Induction of inducible NO synthase (iNOS) mRNA expression in 3T3 fibroblasts after incubation with CM was examined by the reverse transcriptase-polymerase chain reaction (RT-PCR) method.

Results.

Bleomycin induced significant nitrite release from PBMCs in a time-dependent manner. Stimulation with CM increased nitrite production in 3T3 fibroblasts, which was significantly inhibited by antibodies against interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and iNOS inhibitor, L-NMMA. CM from SSc patients induced higher amounts of nitrite from 3T3 fibroblasts, compared with that from normal subjects, although the difference was not significant. CM induced iNOS mRNA expression in 3T3 fibroblasts in a time-dependent manner.

Conclusion.

These results suggest that bleomycin, as well as IL-1β and TNF-α, induce NO release from mononuclear cells, and that these cytokines furthermore stimulate fibroblasts to produce NO, which raises the possibility of the involvement of NO in the development of tissue fibrosis induced by bleomycin.

Introduction

Nitric oxide (NO) has recently been implicated as a mediator of immune and inflammatory responses and has been suggested to play an important role in the regulation of immune function in autoimmune disorders (1). NO can be induced in certain cells, including mononuclear cells, neutrophils, fibroblasts or endothelial cells, by inducible NO synthase (iNOS) after stimulation by inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) or bacterial lipopolysaccharide (LPS) in combination with interferon-γ (IFN-γ) (2).

Systemic sclerosis (SSc) is a connective tissue disease characterized by fibrosis with excessive collagen production and deposition in the skin and various internal organs; however, its pathogenesis remains unknown. We recently showed that the serum NO level is significantly elevated in SSc patients as compared with normal subjects, and an immunohistochemical study demonstrated that scleroderma fibroblasts expressed iNOS protein (3), which suggests the involvement of NO in SSc. A recent report suggests that NO produced by wound fibroblasts may play a role in regulating the process of wound healing (4). However, the role of NO in tissue fibrosis or tissue remodeling is largely unknown.

Bleomycin is one of the causative agents of tissue fibrosis leading to scleroderma-like conditions (5, 6). It is well known that bleomycin induces pulmonary fibrosis, and alveolar macrophages are suggested to play a central role by releasing cytokines that may be involved in inflammation, fibroblast proliferation, and increasing amounts of extracellular matrix (7). We recently observed that bleomycin-stimulated peripheral blood mononuclear cell (PBMC) factors show growth stimulatory activity for fibroblasts *in vitro* (8). To determine the possible involvement of NO in tissue fibrosis, we examined the effect of bleomycin-stimulated PBMCs on the production of nitrite in mouse 3T3 fibroblasts.

Materials and methods

Cells

Mouse balb/c 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's

medium (DMEM) containing 0.58 g L-glutamine, 62.5 μg/ml penicillin G, and 100 μg/ml streptomycin supplemented with 7% heat-inactivated fetal calf serum (FCS). Cells were maintained at 37° C in a humidified atmosphere containing 5% CO_2.

Preparation of conditioned medium

Participants included 6 normal volunteers (3 M and 3 F; mean age: 48.2 years old) and from 6 patients with SSc according to the criteria for SSc proposed by the American Rheumatism Association (ARA). Four had limited cutaneous SSc (lSSc) and 2 had diffuse cutaneous SSc (dSSc) (2 M and 4 F; age range 37 - 60; mean age, 50.6 years old). The mean disease duration was about 4 years, and none of the patients were being treated with systemic steroids or immunosuppressive drugs at the time of blood sampling. Three of them had been treated with vasodilating drugs.

PBMCs were isolated by Ficoll-Paque density gradient centrifugation (Pharmacia, Uppsala, Sweden), resuspended in RPMI 1640 containing 7% FCS, and seeded at 1 x 10^6 cells per well on a 24-well tissue culture plate (Falcon 3047, Becton Dickinson, NJ, USA). On average, the PBMC consisted of 10-20% monocytes and 80-90% lymphocytes in both normal volunteers and SSc patients. Cells were cultured in the presence or absence of bleomycin (100 ng/ml to 10 μg/ml), Concanavalin A (Con A) (Sigma Chemical Co. St. Louis, MO, USA) (5 μg/ml) and staphylococcal enterotoxin B (SEB) (Sigma) (10 ng/ml, decided in a preliminary experiment) for 1 to 3 days. Then the supernatants were centrifuged and collected as conditioned medium (CM). CM was stored at -20°C until use.

Stimulation of fibroblasts and assessment of nitrite

3T3 fibroblasts were seeded on a 24-well plate at 5 x 10^4 in the presence or absence of bleomycin (100 ng/ml to 10 μg/ml) or 10% CM for 1-3 days, and the culture supernatants were harvested, centrifuged and stored at -20°C until use.

In another experiment, 3T3 fibroblasts were incubated with 10% CM with or without antibodies against IL-1β (Cosmo Biological Co. Tokyo, Japan) and TNF-α (Cosmo), and N^G-monomethyl-L-arginine (L-NMMA) (Sigma), a competitive inhibitor of iNOS, for 24 hrs and the supernatant was examined.

Nitrite in the culture supernatants was measured using a colorimetric assay kit (Cayman Chemical Co. MI, USA) based on the Griess reaction. Briefly, 100 μl of cell-free supernatants were incubated with 50 μl of 2.5% phosphoric acid in diluted water and 50 μl of a solution consisting of 1.0% sulfanilamine and 0.1% naphthylethylenediamine dihydrochloride in H_2O for 10 min at room temperature, and the absorbance at 540 nm was determined. The concentration of nitrite was determined by a sodium nitrite standard curve.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

3T3 fibroblasts (1 x 10^5) were seeded on a 6-well plate (Corning, NY, USA) with or without CM (10%) for 6 - 24 hrs. Then, total RNA was isolated using RNA zol (Chinna/Biotex, Houston, TX, USA). 100 ng of extracted RNA was reversely transcribed to cDNA by RAV-2 reverse transcriptase (Takara, Tokyo, Japan). PCR analysis of iNOS was accompanied by using oligonucleotide primers specific for mouse iNOS (sense 5' TTCC GAAGTTTCTGGCAGCA 3'; antisense 5' ATAGGAAAAGACTGCACCGAA GAT 3') and β-actin (sense 5' CTCTTT GATGTCACGCACGATTTC 3'; antisense 5' GTGGGCCGCTCTAGGCA C CAA 3') (9). We used the following conditions: 35 cycles each of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. PCR products were electrophoresed in 1.7% agarose gel. The gel was stained with 1% ethidium bromide and visualized under ultraviolet light. To quantify the PCR products, the intensity of each band was measured by a densitometer (EPA-3000, Chemiway, Tokyo) and compared with that of β-actin. PCR was performed using different number of cycles to ensure that amplification occurred in a linear range. For the negative control, total cellular RNA without reverse transcription was used.

ELISA assay of CM

IL-1β and TNF-α concentrations in CM stimulated with bleomycin (10 μg/ml) for 2 days in normal subjects (n = 3) and SSc patients (n = 3) were determined by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Data were expressed as means ± SD. Statistical analysis was performed using the standard Student's t-test and Mann-Whitney U test. A p value of < 0.05 was considered to be significant.

Results

PBMCs produced a low level of nitrite spontaneously or in the presence of Con A and SEB, while bleomycin induced a significantly increased accumulation of nitrite in PBMCs on day 3, as compared with unstimulated and stimulated PBMC by Con A and SEB ($p < 0.05$) (Fig. 1). Con A, SEB and bleomycin all induced a time-dependent increase in nitrite. Bleomycin induced nitrite production on PBMCs in a dose-dependent manner from 100 ng/ml to 10 μg/ml (data not shown). All of the following experiments were performed using CM stimulated with 10 μg/ml bleomycin for 2-day cultures.

The concentration of IL-1β in CM between normal subjects (20.6 ± 3.6 pg/ml) and SSc patients (78.4 ± 14.1 pg/ml) reached a significant difference ($p < 0.01$), whereas the TNF-α level in the CM of normal subjects (35.0 ± 17.2 pg/ml) and SSc patients (55.6 ± 18.6 pg/ml) did not show a significant difference. IL-1β and TNF-α levels in unstimulated PBMCs were below 15.6 pg/ml and 19.6 ± 9.2 pg/ml, respectively, in normal subjects, 60.4 ± 14.0 pg/ml and 30.5 ± 14.8 pg/ml in SSc patients.

The anti-TNF-α and IL-1β antibodies separately or in combination attenuated the increase in nitrite induced by 10% CM derived from normal subjects after 24 hrs (Fig. 2, $*p < 0.05$, $**p < 0.01$). Furthermore, L-NMMA also significantly inhibited the production of nitrite ($p < 0.01$) (Fig. 2). The mean percent of inhibition was 60% by anti-IL-1β, 67.3% by anti-TNF-α, 78.2% by the combination of both antibodies and 75.5% by L-NMMA in experiments using CM derived from normal subjects (n = 3). On

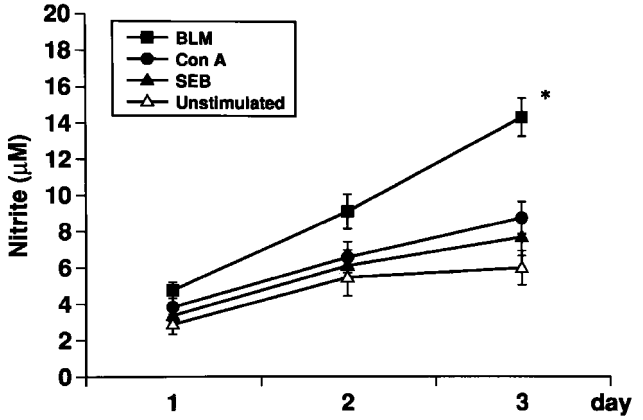

Fig. 1. Production of nitrite by PBMC. PBMC (1 x 10^6) were cultured in the absence or presence of Con A (5 μg/ml) (●), SEB (10 ng/ml) (▲) and bleomycin (10 μg/ml) (■). Nitrite was determined in the supernatant using a colorimetric assay after an incubation period of 1 - 3 days. Error bars present the SD of triplicate assays. *Significant difference between bleomycin-stimulated cells and unstimulated PBMC or stimulated PBMC by Con A and SEB ($p < 0.05$).

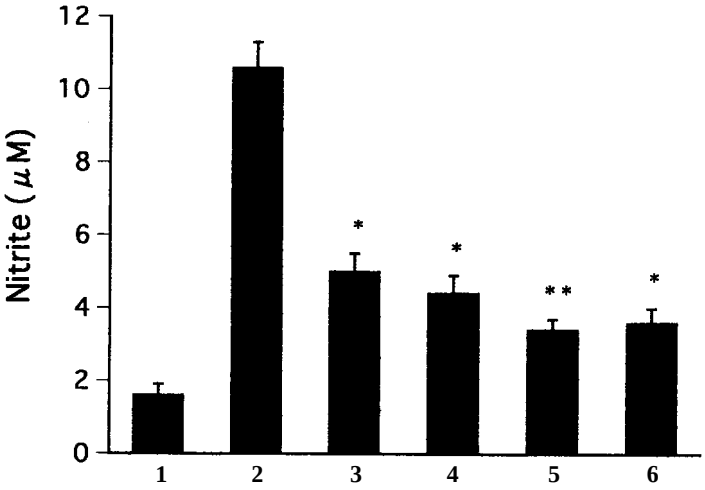

Fig. 2. Effects of anti-IL-1β antibody, anti-TNF-α antibody, and L-NMMA on nitrite production by 3T3 fibroblasts. Increased nitrite production by the addition of CM derived from normal subjects (n = 3) (10%) was attenuated by the incubation of each antibody for IL-1β (5 μg/ml) (*$p < 0.05$), TNF-α (10 μg/ml) (*$p < 0.05$), or combination with IL-1β and TNF-α antibodies (**$p < 0.01$). L-NMMA was also significantly inhibited the nitrite release (*$p < 0.05$). Although representative data of L-NMMA (100 μM) are shown, the dose-dependent inhibitory effects of L-NMMA were exhibited on nitrite production from 3T3 fibroblasts at a dose ranging 10 - 1,000 μM. 1, unstimulated; 2, stimulated with CM; 3, stimulated with CM and anti-IL-1β antibody; 4, stimulated with CM and anti-TNF-α antibody; 5, stimulated with CM, and anti-IL-1β and anti-TNF-α antibodies; 6, stimulated with CM and L-NMMA.

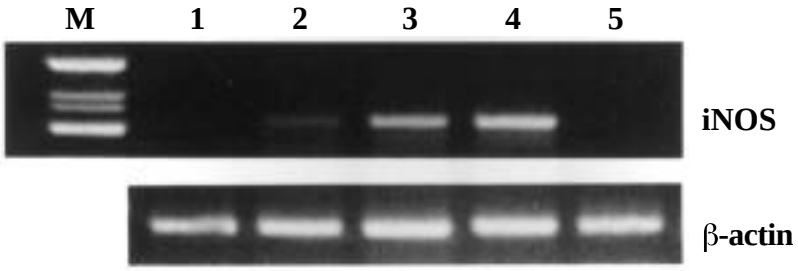

Fig. 3. Induction of iNOS gene expression on 3T3 fibroblasts by the addition of CM. 3T3 fibroblasts (1 x 10^5) were incubated with CM (1: 10 ratio) for 6, 12 and 24 hrs. RT-PCR products were electrophoresed through 1.7% agarose gel and visualized with ethidium bromide. Representative data is shown. (**M**) Marker; lane 1: unstimulated; lane 2: cells incubated with CM for 6 hrs; lane 3: cells incubated with CM for 12 hrs; lane 4: cells incubated with CM for 24 hrs; lane 5: cells incubated with bleomycin for 24 hrs. The iNOS RT-PCR product was 500 bp, the β-actin product was 549 bp.

the other hand, in the experiments using SSc patient-derived CM (n = 3), the mean percent inhibition was 63.2% (IL-1β, 68.5% (TNF-α), 78.6% (both antibodies) and 72.7% (L-NMMA), none of which showed any significant difference between normal subjects and SSc patients. In separate experiments, the anti-TNF-α antibody, anti-IL-1β antibody, and L-NMMA were shown not to decrease the viability determined by trypan blue exclusion of the fibroblasts at the concentrations used up to 24 hrs (viabilities > 95% in all conditions).

CM applied to the cultures of 3T3 fibroblasts induced an increase in nitrite with its peak at 2 days' incubation, whereas addition of bleomycin (100 ng/ml - 10 μg/ml) did not enhance nitrite production in fibroblasts at all during the 1-3 days culture periods (data not shown). Although CM from SSc patients induced a higher amounts of nitrite release in 3T3 fibroblasts (16.1 ± 2.2 μM at 2 days) as compared with that derived from normal subjects (12.0 ± 1.8 μM at 2 days), the difference did not reach significance.

Stimulation with CM (10%) from normal subjects induced iNOS mRNA expression in 3T3 fibroblasts after 6 hrs (Fig. 3). Bleomycin only did not up-regulate the expression of iNOS mRNA in fibroblasts. iNOS mRNA was strongly induced by CM derived from both SSc patients and normal subjects (data not shown).

Discussion

In this study, we demonstrated the potential for bleomycin-stimulated PBMCs to induce nitrite production and iNOS mRNA expression in 3T3 fibroblasts. In contrast, bleomycin itself did not induce nitrite release at all in fibroblasts during the culture periods examined. As the half-life of bleomycin is reported to be about 50 min (10), it is unlikely that the CM used in this study contained bleomycin. The dependence of this increase on TNF-α and IL-1β was shown by the capacity of anti-TNF-α and IL-1β antibodies to decrease nitrite production. Furthermore, iNOS mRNA was induced in fibroblasts as early as 6 hrs after the addition of CM.

NO was originally identified as the endothelium-derived relaxing factor, which

regulates vascular tone. NO is generated endogenously from L-arginine by oxidation to L-citrulline and NO. The release of NO plays an important role in regulating blood flow by inhibiting smooth muscle contraction and platelet aggregation (2, 11). NO is also suggested to be a crucial mediator in inflammatory cascades and to play a role in immunoregulation. Recently, human dermal fibroblasts have been shown to produce NO and to express both cNOS and iNOS mRNAs and proteins (12). Another report demonstrated that NO is synthesized by wound fibroblasts, which are characterized by increased collagen synthesis and constriction (4), suggesting that the NO produced by fibroblasts may play a role in regulating the processes of inflammation and tissue remodeling.

Our data in the present study show that CM derived from SSc patients induced higher amounts of nitrite in 3T3 fibroblasts, as compared with that derived from normal subjects. One reason is that IL-1β and TNF-α levels in CM derived from SSc patients are higher than that from normal subjects. Inflammatory cytokines such as IFN-γ, IL-1, TNF-α or LPS have been shown to induce the expression of NOS in certain cells *in vitro.* In contrast, transforming growth factor-β (TGF-β), which is supposed to play a key role in the fibrotic process, has been shown to inhibit NO synthesis by reducing both NOS mRNA and protein stability in monocytes (13). However, recent findings have shown that TGF-β alone does not have any detectable effect on NO production, but increases NO synergistically with IL-1β in chondrocytes (14). Further studies are necessary to clarify the precise role of NO in the pathogenesis of scleroderma. Bleomycin is an antibiotic obtained from *Streptomyces venticillus* and has been found to be effective for the treatment of certain neoplasms. Bleomycin is known to be capable of inducing pulmonary fibrosis or scleroderma-like conditions including Raynaud's phenomenon in patients with cancers (5, 6). On the other hand, previous studies have shown that macrophages play a central role by releasing mediators that may be involved in bleomycin-induced pulmonary fibrosis (7). Our recent observation also indicated that bleomycin-stimulated PBMCs show growth stimulatory activity for fibroblasts (8). Monocyte activation has been previously reported in patients with SSc (15). Our data presented here show that bleomycin stimulates PBMCs to release significant amounts of nitrite as well as IL-1β and TNF-α, which was suggested to further act on fibroblasts to induce nitrite production via IL-1β or TNF-α. Increased amounts of NO may play a role in the induction of Raynaud's phenomenon. It is reported that when produced in excess, NO or its reaction products may be cytotoxic to host cells (16). Although *in vitro* data may not correctly reflect on the *in vivo* situation, these results indicate that PBMCs are important in regulating NO production when exposed to bleomycin by releasing cytokines which stimulate NO release, suggesting a potential mechanism for the regulation of NO production in the tissue fibrosis by bleomycin.

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